miR-29a maintains mouse hematopoietic stem cell self-renewal by regulating *Dnmt3a*

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properties are incompletely understood. Herein, we show that homozygous deletion of the *miR-29a/b-1* bicistron results in decreased numbers of hematopoietic stem and progenitor cells (HSPCs), decreased HSC self-renewal, and increased HSC cell cycling and apoptosis. The HSPC phenotype is specifically due to loss of *miR-29a*, because *miR-29b* expression is unaltered in *miR-29a/b-1*-null HSCs, and only ectopic expression of *miR-29a* restores HSPC function both in vitro and in vivo. HSCs lacking *miR-29a/b-1* exhibit widespread transcriptional dysregulation and adopt gene expression patterns similar to normal committed progenitors. A number of predicted *miR-29* target genes, including *Dnmt3a*, are significantly upregulated in *miR-29a/b-1*-null HSCs. The loss of negative regulation of *Dnmt3a* by *miR-29a* is a major contributor to the *miR-29a/b-1*-null HSPC phenotype, as both in vitro *Dnmt3a* short hairpin RNA knockdown assays and a genetic haploinsufficiency model of *Dnmt3a* restored the frequency and long-term reconstitution capacity of HSCs from *miR-29a/b-1*-deficient mice. Overall, these data demonstrate that *miR-29a* is critical for maintaining HSC function through its negative regulation of *Dnmt3a*. (*Blood*. 2015;125(14):2206-2216)

Introduction

Hematopoietic stem cells (HSCs) are the only cells in the blood system capable of giving rise to all mature hematopoietic cells while self-renewing for the lifetime of an organism.[1,2] Although HSCs have been extensively characterized with respect to expression of their protein-encoding transcripts,[3,4] relatively little is known about the role of non–protein-encoding RNAs in HSC function, particularly microRNAs (miRNAs). miRNAs are small noncoding RNAs (22-24 nts) that exert their biological effects by negatively regulating the stability and/or translation efficiency of multiple target mRNAs.[5] Given the ability of each miRNA to target hundreds of mRNAs on average, it is not surprising that miRNAs play critical roles in regulating gene networks involved in stem cell self-renewal, development, and carcinogenesis.[6,7]

Our laboratory and others have performed miRNA expression profiling studies using highly purified HSCs and committed progenitors in both the mouse and human hematopoietic systems and demonstrated that different hematopoietic stem and progenitor cell (HSPC) populations exhibit unique miRNA expression patterns, supporting a role for miRNAs in their distinctive biological functions.[8-11] For example, miRNAs expressed at high levels in HSCs such as *miR-125a/b*,[12-14] *miR-126*,[15] and *miR-146a*[16] regulate HSC self-renewal or differentiation. More recently, *miR-29a* was shown to play an important role in directly regulating innate and adaptive immune responses[17] by targeting interferon-γ[18] or indirectly by protecting the thymus from inappropriate involution through suppression of the interferon-α receptor.[19] The *miR-29* family is also more broadly relevant to stem cell biology, as *miR-29b* recently was shown to be upregulated by *Sox2* and required for reprogramming of fibroblasts into induced pluripotent stem cells.[20] Although little is known about how *miR-29a* transcript levels are regulated, CCAAT/enhancer-binding protein alpha (CEBPA) has been reported to positively regulate the *miR-29a/b-1* cluster and expression of *miR-29a/b* is suppressed in acute myeloid leukemia (AML) patients with impaired CEBPA function.[21]

To understand the physiologic role of *miR-29a* in normal hematopoietic development, we evaluated HSPCs in mice harboring a genetic deletion of the *miR-29a/b-1* bicistron.[19] Here we demonstrate that deletion of the *miR-29a/b-1* bicistron results in decreased HSC self-renewal and long-term reconstitution capacity. This loss of HSC function is associated with increased HSC cell cycling and apoptosis, as well as acquisition of a gene expression profile similar to more differentiated hematopoietic progenitors. Among the differentially

expressed transcripts are multiple predicted *miR-29a* targets including *Dnmt3a*. By crossing *miR-29a/b-1* heterozygous mice to *Dnmt3a* heterozygous mice, we show that the functional defects observed in *miR-29a*-null HSCs are largely mediated via abrogation of its negative regulation of *Dnmt3a* expression. Overall, these studies indicate that *miR-29a* is essential in maintaining HSC function and mediates its effects by modulating the activity of the epigenetic regulator *Dnmt3a*.

Materials and methods

Retrovirus preparation and transduction

The *Dnmt3a* short hairpin RNA (shRNA) constructs were cloned into the pMig plasmid and were gifts from Dr Iannis Aifantis (New York University, New York, NY). Retroviral preparation and donor cell infections/transplantations were performed as previously described.[9] The ability of 2 shRNA clones (197 and 6567) to knock down *Dnmt3a* was confirmed in NIH/3T3 cells. DNMT3a antibody (2160S; Cell Signaling) was used to confirm the protein levels after knockdown.

Mice/transplantations

The generation of *miR-29a/b-1*-null mice was previously described.[19] Donor and recipient mice (C57BL/6 and B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ, respectively) were bred and maintained in the Memorial Sloan Kettering Cancer Center mouse facility. *Dnmt3a*$^{wt/fl}$[Mx-Cre] mice were crossed with *miR-29a/b-1* Het mice, and their progeny were injected intraperitoneally 6 times with 300 μg polyinosinic: polycytidylic acid (Sigma) in phosphate-buffered saline every other day to induce deletion of *miR-29a/b-1* floxed alleles. All progeny contained Mx-Cre knockin to diminish the bias from Cre expression. Recipients were retro-orbitally transplanted following lethal irradiation using a γ radiation source (9.5 Gy total) and maintained on antibiotics (Sulfatrim) for 6 weeks following transplantation. Total bone marrow cells (2 million donor cells) or magnetic bead-enriched (Miltenyi Biotec) c-Kit^{+} cells (500 000 cells) were used for noncompetitive or competitive transplants, respectively. Competitive transplantations were performed with equal numbers of competitor bone marrow cells. Following transplant, the peripheral blood was sampled monthly to evaluate donor chimerism and lineage composition. All mouse procedures were performed in accordance with institutional guidelines as described in an Institutional Animal Care and Use Committee (IACUC) approved protocol.

miRNA expression analysis

The expression of *miR-29a*, *miR-29b*, and *miR-29c* was measured using a QuantiMir kit per the manufacturer's instructions (System Biosciences). Synthesized oligonucleotides containing mature miRNA sequences were used as primers for *miR-29* family member genes. Total RNA was prepared from total or c-Kit^{+}-enriched bone marrow cells using the RNeasy Mini Kit (Qiagen). Mouse snoRNA202 was used as an endogenous control to normalize for total RNA loaded.

Methylcellulose colony forming assays

To evaluate self-renewal and proliferation of HSPCs, fluorescence-activated cell sorter (FACS)-purified c-Kit^{+} HSPCs were cultured in methylcellulose medium supplemented with cytokines (Methocult GF M3434; Stem Cell Technologies). Colony numbers were counted 12 days after plating. Serial replating cultures were performed by harvesting cells from methylcellulose media, followed by plating 20 000 cells in fresh methylcellulose.

Cell staining and flow cytometry

Mouse bone marrow cells were harvested and stained as previously described.[22] Briefly, antibodies used in this study include a lineage (Lin) cocktail containing antibodies against Ter-119 (clone Ter-119), B220 (RA3-6B2), CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7), Gr-1 (RB6-8C5), and Mac-1 (M1/70) antibodies, conjugated with either phycoerythrin (PE)-Cy5 or PE-Cy7 (eBiosciences). Additional antibodies used to identify HSPCs included CD16/32 (93) in Alexa eFluor 700, IL7Ra (A7R34) in PE-Cy5, CD45.1 (A20) in PE-Cy7, c-Kit (2B8) in allophycocyanin (APC)-eFluor 780, Gr-1 (RB6-8C5) in PE, CD45.2 (104) in Alexa eFluor 700, CD34 (RAM34) in fluorescein isothiocyanate, Flk2/Flt3 (A2F10) in PE (all from eBiosciences), as well as Sca-1 (E13-161.7) in Pacific Blue and CD150 (TC15-12F12.2) in PE (both from BioLegend). After staining, cells were analyzed and sorted using a FACSAria II (Becton Dickinson). All cell populations were double sorted, and a purity of >90% was routinely achieved. Flow cytometry data were analyzed using Flowjo software (TreeStar, Inc.).

Cell cycle and apoptosis analysis

For cell cycle analysis, bone marrow cells were stained with antibodies to identify HSPCs as described above and then fixed using a Fixation/Permeabilization Kit (eBioscience) and stained with 4,6 diamidino-2-phenylindole (DAPI; Invitrogen). 5-Bromo-2′-deoxyuridine (BrdU) retention assays were performed by analyzing bone marrow cells 20 hours after intraperitoneal injection with 150 μL BrdU (10 mg/mL) per mouse (BD Pharmingen). A fluorescein isothiocyanate-conjugated anti-BrdU antibody was used to detect BrdU content by flow cytometry. Apoptosis was evaluated using an Annexin V staining kit per the manufacturer's instructions (BD).

Microarray analysis

Total RNA was extracted from double FACS-sorted cell populations using an RNeasy Mini Kit (Qiagen), Nugen Ovation Pico WTA was used for preamplification of RNA samples and was hybridized to Illumina Mouse 6 arrays using standard protocols. Array data were analyzed using open source R/Bioconduct software including lumi and limma packages (R Development Core Team, 2012). The microarray data were submitted to the Gene Expression Omnibus (GEO) database (accession number GSE58237). Predicted *miR-29a/b-1* target genes were identified using the online database targetscan.org.[23]

Data analysis and statistics

Data were summarized and presented using open source R/Bioconductor software (R Development Core Team, 2012). Statistical tests were performed using the appropriate software packages accompanying this program, including a 2-tailed *t* test between groups (*$P < .05$; **$P < .01$); log-rank tests were used for comparison of Kaplan-Meier curves.[24] Error bars in bar graphs indicate standard error of the mean (SEM).

Results

miR-29a/b-1 positively regulates hematopoietic stem/progenitor cell number

To determine the role of *miR-29a/b-1* in hematopoiesis, we assessed the frequencies of HSPCs in mice harboring a constitutive deletion of the *miR-29a/b-1* bicistronic cluster on chromosome 6 by flow cytometry (FC).[19] In adult mice (12-16 weeks old), deletion of *miR-29a/b-1* resulted in significantly decreased frequencies of Lin^{-}c-Kit^{+}Sca-1^{+} (LSK) and Lin^{-}c-Kit^{+}Sca-1^{-} (committed progenitors) cells compared with wild-type (WT) littermates (Figure 1A,C,E). *miR-29a/b-1*$^{-/-}$ mice also exhibited a 20.1% decrease in bone marrow cellularity (Figure 1B). Furthermore, deletion of *miR-29a/b-1* resulted in significantly reduced frequencies of populations within the LSK population (HSCs and multipotent progenitors [MPPs]), as well as in committed progenitors (common myeloid progenitor [CMP], granulocyte-macrophage progenitor [GMP], megakaryocyte-erythroid progenitor [MEP], and common lymphoid progenitor [CLP]; Figure 1A,D,F-G). Despite these alterations in HSPC composition, *miR-29a/b-1*$^{-/-}$ mice did not exhibit statistically significant differences in their peripheral blood counts compared with those of their WT littermates (supplemental Figure 1, available on the *Blood* Web site). In addition, FC analysis of the peripheral blood failed to demonstrate differences in the

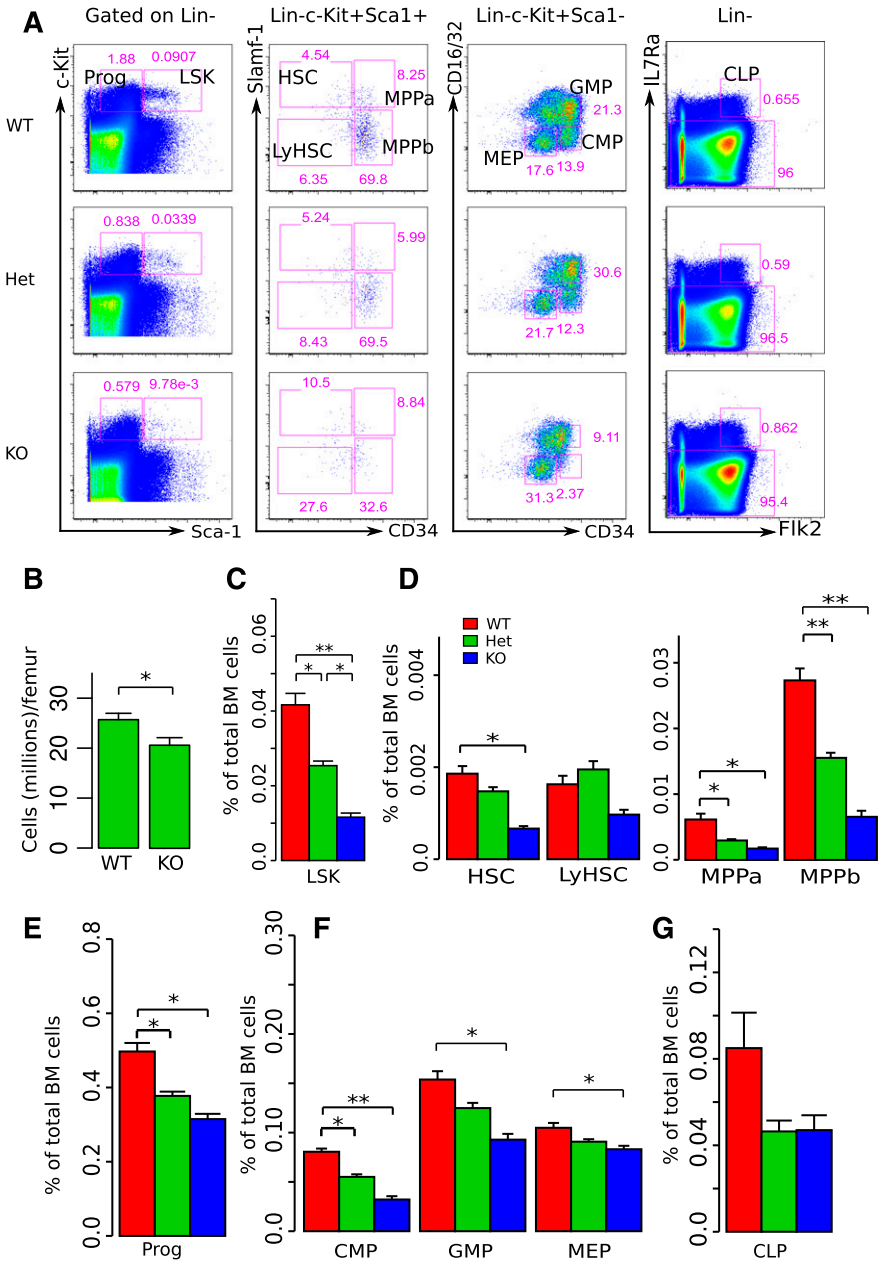

Figure 1. *miR-29a/b-1*-null mice exhibit decreased bone marrow cellularity and significantly reduced numbers of HSPCs. (A) Representative flow cytometric analyses. Each column includes WT littermate, *miR-29a/b-1* Het, and *miR-29a/b-1*-null (KO) mice. Live cells were gated based on propidium iodide exclusion. Prior gates for each subpopulation are indicated on the top of each column. Specific HSPC populations and their frequencies were calculated based on total bone marrow cell counts and were identified as shown. (B) Total bone marrow cell counts were based on bilateral femur cell recoveries. (C-G) Summarized results of bone marrow HSPC frequencies assessed by flow cytometry. The cell populations evaluated were indicated at the x-axis, and the y-axis represents the frequencies of these populations among total bone marrow cells. Each group included 7 to 10 mice. Error bars indicated SEM. Statistical significance was calculated using a Student *t* test: $^{*}P < .05$; $^{**}P < .01$.

frequencies of myeloid or T cells in *miR-29a/b-1*$^{-/-}$ mice compared with WT littermate controls, although *miR-29a/b-1*$^{-/-}$ mice did demonstrate a significant decrease in B220^{+} B cells (supplemental Figure 2). To determine whether alterations in HSPC composition are specific to adult hematopoiesis, we evaluated fetal liver hematopoiesis in E13.5~E14.5 *miR-29a/b-1*$^{-/-}$ embryos. There was no difference in HSPC frequencies or total cellularity in fetal liver, indicating a developmental stage-specific effect of *miR-29a/b-1* function (supplemental Figure 3A-D). These data indicate that loss of *miR-29a/b-1* results in a significant reduction in the number of HSPCs during steady-state adult hematopoiesis.

Loss of *miR-29a/b-1* results in impaired stem/progenitor cell colony forming capability

To determine whether the reduction in HSPC numbers in *miR-29a/b-1*-deficient mice is due to decreased HSPC self-renewal and/or proliferative capacity, we performed methylcellulose colony formation assays with serial replating. *miR-29a/b-1*$^{-/-}$ cells gave rise to fewer and smaller colonies compared with WT littermate controls when 3000 c-Kit^{+} cells were plated into methylcellulose media (Figure 2A-B). The reduction in colony forming activity in *miR-29a/b-1*$^{-/-}$ cells compared with WT controls was more severe in secondary plating experiments, and by the third plating, too few cells were generated from *miR-29a/b-1*$^{-/-}$ cells to be replated (Figure 2A).

To investigate the effect of *miR-29a/b-1* loss on specific HSPC populations, we performed colony formation assays using FACS-sorted HSC, MPP, CMP, GMP, and MEP cells.[25-28] All these *miR-29a/b-1*-deficient populations exhibited significant reductions in colony numbers with the exception of GMPs (Figure 2C), but all HSPC populations, including GMPs, gave rise to smaller colonies. The differences in colony formation were more pronounced in cultures initiated by the most immature populations (ie, HSCs and MPPs; Figure 2C). Together, these data indicate that *miR-29a/b-1* positively regulates HSPC proliferation and self-renewal capacity.

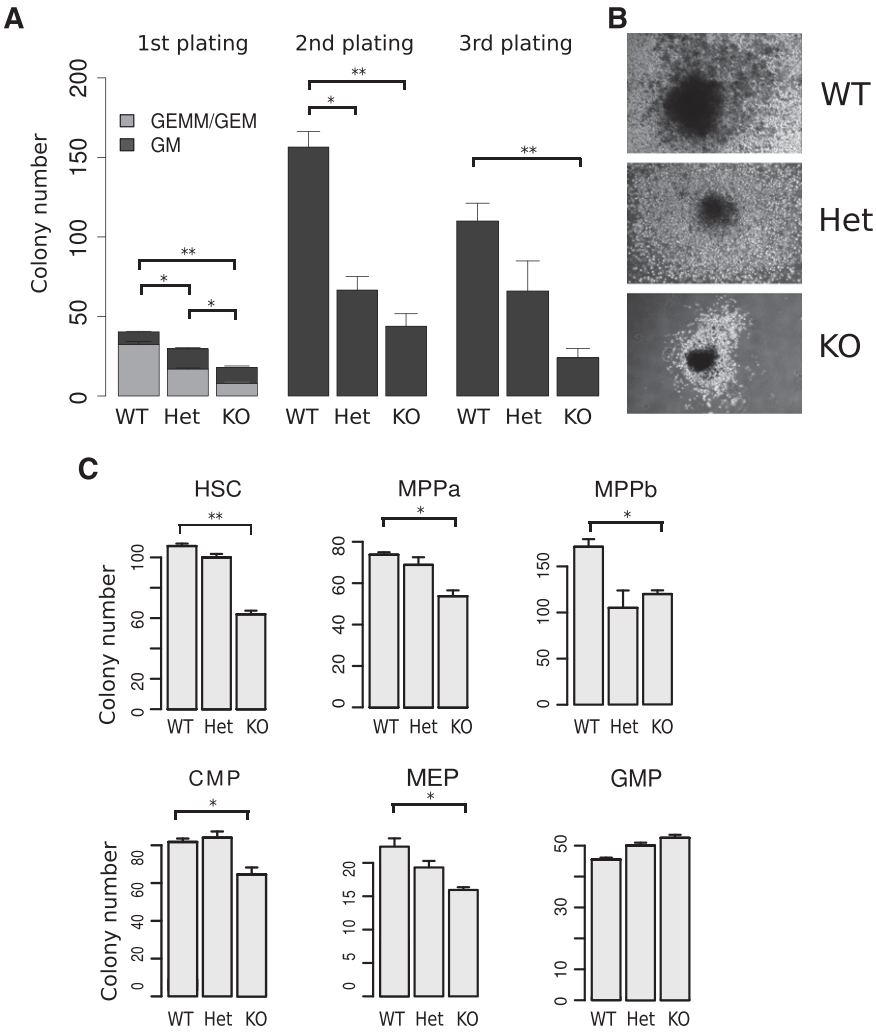

Figure 2. *miR-29a/b-1*-null HSPCs exhibit decreased colony forming capacity in methylcellulose colony assays. (A) Methylcellulose colony assays were performed using c-Kit$^+$-enriched bone marrow cells. The first plating was initiated using 3000 c-Kit$^+$ cells from WT, Het, and KO mice. Replatings were performed using 20 000 cells per well. (B) Photomicrographs of typical colonies generated by WT, Het, and KO HSPCs on initial plating. (C) Methylcellulose colony assays were performed using purified HSPCs. Six populations, including HSC, MPPa, MPPb, CMP, MEP, and GMP, were double FACS sorted and 150 (HSC, MPPa, MPPb) or 400 (CMP, MEP, and GMP) cells were plated. Data shown represent the aggregate of 3 technical replicates. Error bars indicate SEM. Statistical significance was calculated using a Student *t* test: $^*P < .05$; $^{**}P < .01$.

miR-29a/b-1-null HSCs exhibit cell-intrinsic defects in self-renewal in vivo

To further examine the self-renewal defect of *miR-29a/b-1*-null HSCs, as well as to determine whether the hematologic phenotype is cell intrinsic, long-term reconstitution assays were performed using total bone marrow cells, as well as partially purified HSPC populations. Lethally irradiated mice (CD45.1, B6.SJL-Ptprc^a Pepc^b/BoyJ) were transplanted with 2 million unfractionated, CD45.2-positive bone marrow cells from *miR-29a/b-1*-null (KO), *miR-29a/b-1*$^{+/-}$ (Het), or WT littermate controls. Donor chimerism was monitored serially in transplanted recipients by sampling the peripheral blood. Although WT and Het bone marrow cells gave rise to lymphoid and myeloid progeny at similar frequencies (92.3% and 85.6% donor chimerism, respectively, at week 4 after transplantation), KO bone marrow cells reconstituted transplanted recipients poorly (28.5% donor chimerism at week 4), and their chimerism levels decreased progressively over time (Figure 3A-E).

One possible explanation for the decreased engraftment capacity of KO HSCs is that they possess intrinsic defects in their bone marrow homing capacity. To examine this possibility, we competitively transplanted 500 000 c-Kit$^+$ cells from WT, Het, and KO mice (CD45.2) into lethally irradiated congenic recipients (CD45.1), along with 500 000 total bone marrow cells expressing the recipient CD45.1 allele. After 20 hours, mice were euthanized, and bone marrow cells were evaluated for the presence of donor cells. Equal numbers of KO, Het, and WT donor HSCs (Lin$^-$Sca-1$^+$c-Kit$^+$Slamf1$^+$) were present in the bone marrows of recipients, thereby providing no support for a homing defect (Figure 3F).

Analysis of long-term engrafted mice revealed that transplanted KO cells contributed to HSPCs at lower levels than WT littermate controls (Figure 3G-H). Similar deficits in HSPC engraftment and reconstitution were observed when 500 000 c-Kit$^+$ bone marrow cells from KO mice were competitively transplanted with 500 000 WT total bone marrow cells (supplemental Figure 4A-E). Het donor chimerism in the peripheral blood was also significantly lower than WT littermates when transplantations were performed competitively. These data indicate that *miR-29a/b-1* regulates HSC long-term reconstitution capacity.

To further characterize the defects in *miR-29a/b-1* Het HSPC function, we transplanted total bone marrow cells from noncompetitively engrafted recipients exhibiting high levels of WT or Het donor chimerism (~90%). Secondary transplantation of Het bone marrow cells gave rise to significantly lower levels of CD45.2 donor chimerism than WT cells (Het 11.5% vs WT 72.3% at 4 weeks; $P < .05$), with Het donor chimerism levels remaining low over time (Figure 3I-M). Both LSK and committed progenitor cells from Het HSPC transplanted recipients were present in the BM of secondary recipients at significantly lower frequencies at 16 weeks after transplantation compared with recipients receiving WT cells (Figure 3N), consistent with a gene dosage-dependent role of *miR-29a/b-1* in sustaining HSC self-renewal.

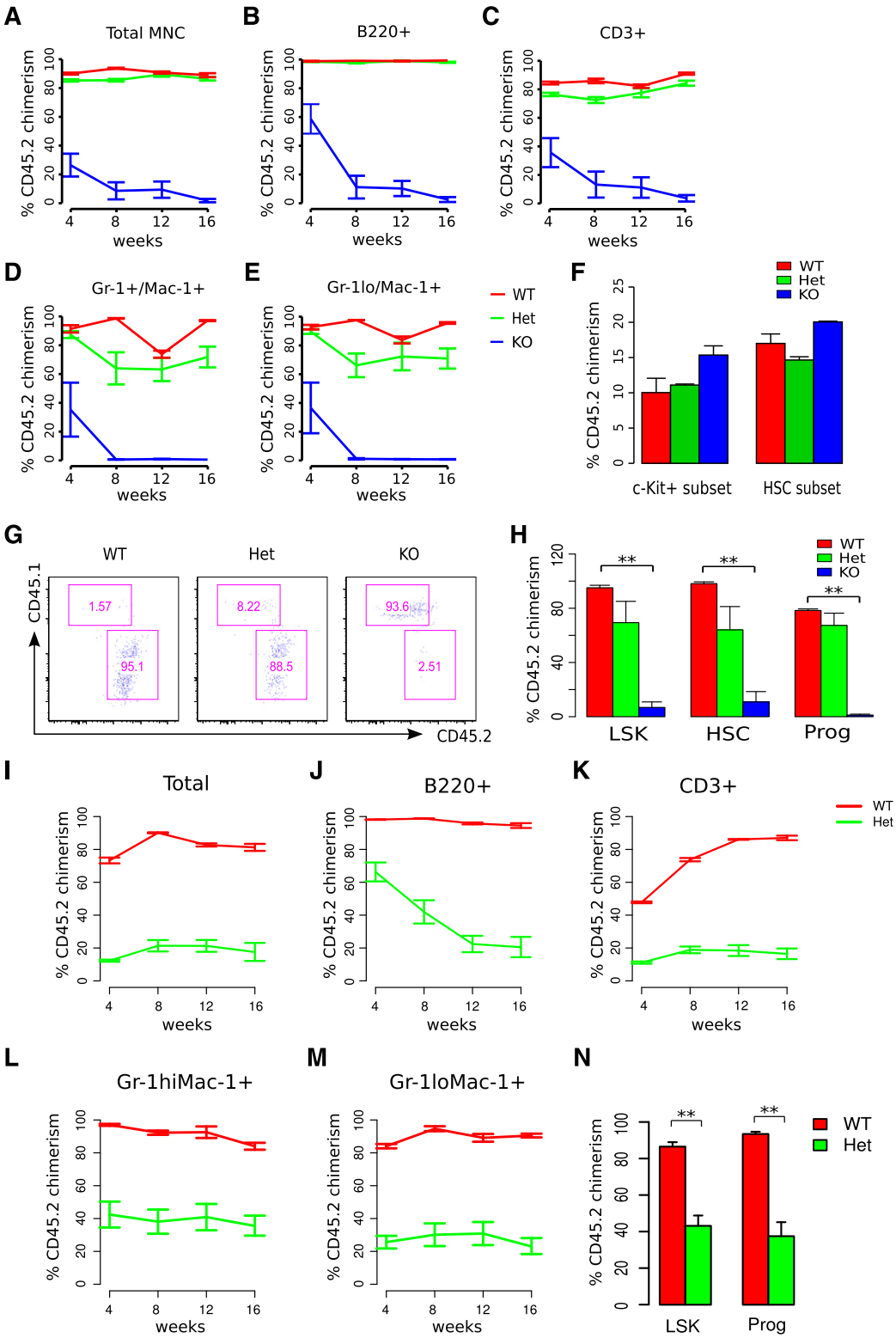

Figure 3. *miR-29a/b-1*-deficient HSCs exhibit reduced self-renewal and reconstitution capacity. (A-E) Donor cell chimerism of total peripheral blood leukocytes or specific cell lineages in the peripheral blood was examined following total bone marrow cell transplant. Donor chimerism ($CD45.2^+$) levels were evaluated following transplantation of 2 million total bone marrow cells from WT, Het, or KO mice into lethally irradiated recipients. (F) Bone marrow cells from both femurs and tibias were examined for donor chimerism 20 hours after transplant using the same transplantation protocol used for competitive transplants. Each group includes 6 to 10 recipient mice transplanted with cells from >3 different donors. (G) Representative flow cytometric analysis of HSPCs in WT, Het, and KO recipients 16 weeks after transplant, previously gated on LSK cells. (H) Summarized results of flow cytometric analysis of bone marrow cells in recipients 4 months after the second transplantation. (I-M) Donor cell chimerism of secondary recipients transplanted with total bone marrow cells from primary recipients transplanted with WT or Het cells. (N) Flow cytometric analysis of HSPCs in the bone marrow of secondary recipients. Statistical significance was calculated using a Student *t* test: $*P < .05$; $**P < .01$.

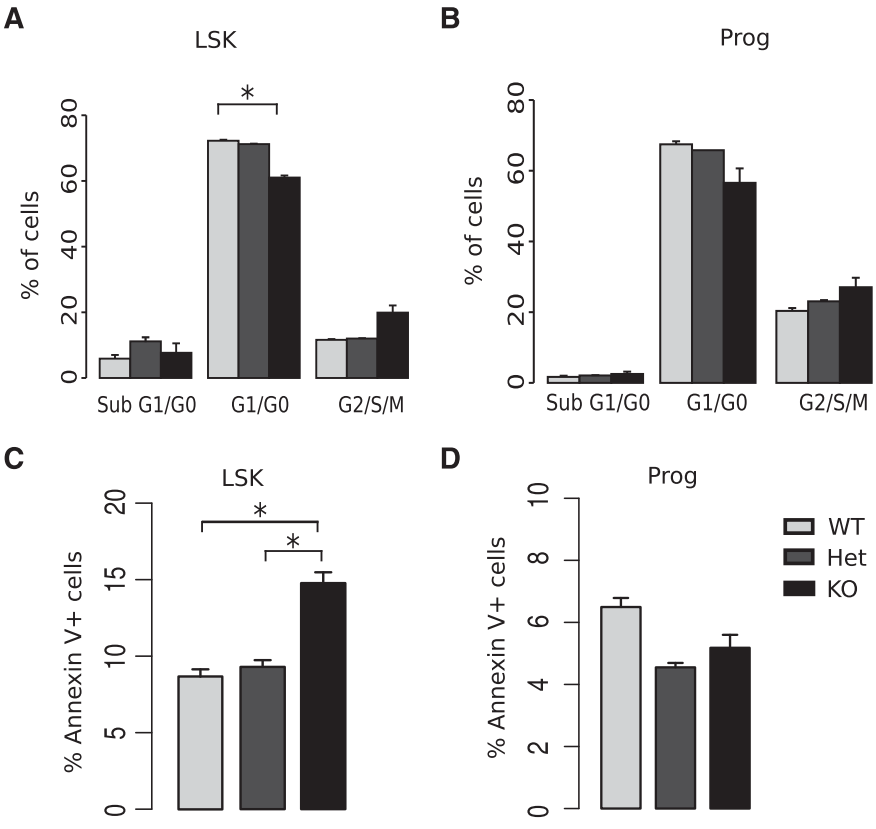

Figure 4. Deletion of *miR-29a/b-1* is associated with increased HSPC cell cycling and apoptosis. (A-B) Cell cycle analysis by staining DNA content of fixed cells with DAPI. Fewer LSK cells, but not committed progenitor cells, accumulated in the G1/G0 stages of the cell cycle in *miR-29a* KO mice. (C-D) Increased numbers of annexin V^+ LSK cells, but not myeloid progenitor cells, were present in both *miR-29a* KO and Het mice compared with WT. Details of annexin V staining and cell cycle staining for specific HSPC populations are summarized in supplemental Figures 5 and 7. Error bars indicate SEM. Each group has 7 to 10 mice from different experiments. Statistical significance was calculated using a Student *t* test: $*P < .05$; $**P < 0.01$.

miR-29a/b-1 maintains HSC quiescence and promotes cell survival

Because HSC quiescence is highly associated with self-renewal capacity,[29] we evaluated the cell cycle status of KO HSPCs. Although KO LSK cells exhibited a significantly higher percentage of cells in the S-G2-M phases of the cell cycle compared with WT littermate LSK cells (Figure 4A; supplemental Figure 5), KO committed progenitors did not exhibit alterations in cell cycle distribution (Figure 4B). To confirm that these findings reflected differences in cell cycle progression, we assessed cell cycle entry using BrdU incorporation assays and found that LSK cells from KO mice cycled more rapidly than WT littermate controls (41.63% vs 58.64% in G_0, respectively; $P < .05$; supplemental Figure 6). As decreased HSC number and reduced bone marrow cellularity may be due to increased apoptosis, we performed annexin V staining. KO mice exhibited a higher frequency of apoptotic LSK cells (annexin V^+7-aminoactinomycin D (7-AAD)$^-$) than WT littermates. In contrast, there was no difference in apoptosis observed in committed progenitors (Figure 4C-D; supplemental Figure 7). Together, these results demonstrate that *miR-29a/b-1* loss impairs the maintenance of quiescence and cell survival specifically in LSK cells, which are composed of the most immature hematopoietic cells including HSCs and MPPs.

miR-29a/b-1-null hematopoietic phenotype is due to *miR-29a* loss

As the *miR-29a/b-1* KO mouse harbors a deletion of 2 miRNAs, and the *miR-29* family is represented by 2 bicistrons including *miR-29a/miR-29b-1* on chromosome 7 and *miR-29b-2/miR-29c* on chromosome 1, we sought to determine which *miR-29* family member is responsible for the *miR-29a/b-1* KO HSPC phenotype. By quantitative real-time polymerase chain reaction (qRT-PCR), we confirmed that *miR-29a* expression was absent in c-Kit^+ cells from KO mice, whereas *miR-29b* and *miR-29c* expression were not significantly altered (Figure 5A). Using retroviruses expressing *miR-29a* or *miR-29b*, only ectopic overexpression of *miR-29a* in KO LSKs significantly increased colony numbers (Figure 5B). Finally, the ability of *miR-29a* to reconstitute the function of KO LSK cells was confirmed in transplantation assays in which overexpression of *miR-29a,* but not *miR-29b,* restored KO donor cell chimerism in a multilineage fashion (Figure 5C; data not shown). Together, these data indicate that *miR-29a* is responsible for the functional defects observed in the KO mice, which we now refer to *miR-29a*-deficient mice.

miR-29a promotes HSC differentiation by regulating multiple target genes

In silico algorithms predict that *miR-29a* targets several hundred mRNAs (eg, targetscan.org).[7] However, the interactions between miRNAs and their target genes depend on their relative expression levels.[30] To better understand the molecular pathways altered due to loss of *miR-29a* in HSCs, we performed gene expression profiling of FACS-purified HSCs (Lin^-c-Kit^+Sca-$1^+CD34^-Slamf1^+$) and committed progenitors (Lin^-c-Kit^+Sca-1^-) from *miR-29a* KO mice. Using a *P* value cutoff of $<.05$, our analysis showed that *miR-29a/b-1* homozygous deletion resulted in dysregulated expression of a larger number of genes in HSCs (1238 genes) than in committed progenitor cells (772 genes; Figure 6A). Unsupervised hierarchical clustering analysis revealed that the KO HSCs exhibit an altered gene expression profile compared with WT HSCs, and their expression patterns are more closely related to WT committed progenitors than WT HSCs (Figure 6B). Gene set enrichment analysis[31] demonstrated a significant enrichment of cell cycle regulators in KO HSCs, consistent with their

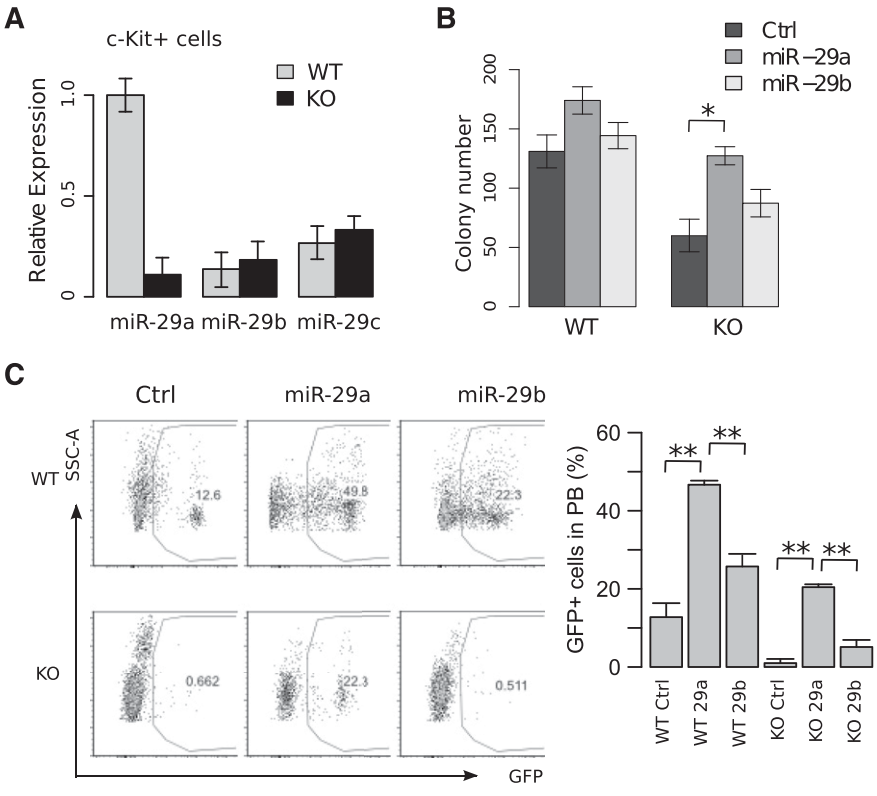

Figure 5. Overexpression of *miR-29a*, but not *miR-29b*, rescues the self-renewal defect in *miR-29a/b-1*-null HSCs. Transcript levels of *miR-29* members were measured in (A) c-Kit-enriched cells. (B) Methylcellulose colony forming capability of LSK cells after retroviral overexpression of *miR-29a* or *miR-29b* in *miR-29a/b-1*-null HSPCs. Twenty-four hours following transduction, GFP$^+$ cells were sorted, and 300 were plated per well. Each group represents experiments using 2 mice, with wells plated in triplicate. (C) Transplantation of 300 000 LSK cells from WT or *miR-29a/b-1*-null HSPCs retrovirally transduced with *miR-29a*, *-29b*, or GFP control (n = 6-10 mice per group). Peripheral blood analysis was performed 16 weeks after transplantation. Data represent the aggregate of 2 independent experiments. Error bars indicate SEM. Statistical significance was calculated using a Student *t* test: $^*P < .05$.

altered cell cycle status (supplemental Figure 8). In an unsupervised hierarchical clustering analysis considering predicted *miR-29a* target genes,[7,23] we identified a group of these genes similarly expressed in WT and *miR-29a*-null committed progenitors, but significantly upregulated in *miR-29a*-null HSCs compared with WT HSCs. These genes included *Dnmt3a*, *Diablo*, *Spast*, *Cdc7*, *Clk2*, *Kdelc1*, *Nasp*, *Ywhae*, *Calm3*, *Larp4*, and *Chmp6* (Figure 6C). To validate our microarray results, we performed qRT-PCR to measure the expression of dysregulated *miR-29a* target genes and additional genes that regulate HSC function using c-Kit$^+$ bone marrow cells from the KO and WT littermates. We confirmed the increased expression of predicted *miR-29a* targets in KO cells, as well as additional genes that regulate HSC function such as *p21*, *p27*, *Bmi1*, *Gata2*, and *TP53* (Figure 6D-E).

miR-29a regulates HSC function in part through inhibition of *Dnmt3a*

Transcriptional profiling revealed that *Dnmt3a* transcript levels were increased 1.55-fold in *miR-29a*-null HSCs compared with WT HSCs ($P = .03$), and this result was confirmed by qRT-PCR (Figure 6D). As *miR-29* has been shown to target DNA methyltransferase 3a (*Dnmt3a*) in the context of cancer,[32,33] and *Dnmt3a* plays a prominent role in maintaining HSC function and lineage commitment[34] and is frequently mutated in acute myeloid leukemia,[35-37] we investigated whether *Dnmt3a* is required for the *miR-29a*-deficient HSPC phenotype. To test whether *Dnmt3a* deficiency can restore normal function in *miR-29a*-null HSCs, we knocked down *Dnmt3a* in LSK cells using 2 retroviral shRNAs (196 and 6567) that had been previously validated in NIH/3T3 cells (Figure 7A; supplemental Figure 9). After *Dnmt3a* shRNA-transduced LSK cells (GFP$^+$) were double FACS-sorted and plated into methylcellulose media, KO LSK cells demonstrated a significant increase in colony formation capacity compared with vector controls (Figure 7B).

To confirm that *miR-29a* and *Dnmt3a* functionally interact in vivo, we crossed mice deficient in *miR-29a* and *Dnmt3a* and found that LSK cell frequencies were similar in WT and Het *Dnmt3a* mice, and *miR-29a* Het LSKs were significantly decreased compared with both WT and Het *Dnmt3a* mice. Mice heterozygous for both *Dnmt3a* and *miR-29a* exhibited significantly higher frequencies of LSK cells than *miR-29a* Het mice, but committed progenitor cell frequencies were unaltered (Figure 7C). This restoration of *miR-29a* Het LSK cell number on *Dnmt3a* loss was associated with increased quiescence, supporting a central role of *Dnmt3a* in the cell cycle alterations observed in *miR-29a* KO HSCs (Figure 7D). *Dnmt3a* mRNA expression was upregulated in *miR-29a* heterozygous LSK cells, and *Dnmt3a* mRNA levels were restored to WT levels in mice compound heterozygous for *Dnmt3a* and *miR-29a* (supplemental Figure 10A). In contrast, *miR-29a* expression levels were unaltered in *Dnmt3a*-deficient LSK cells (supplemental Figure 10B). Together, these data indicate that *Dnmt3a* expression is regulated by *miR-29a*, and that de-repression of *Dnmt3a* expression in the context of *miR-29a* contributes to the defects observed in *miR-29a* Het and KO HSCs.

To determine whether *Dnmt3a* reduction can restore *miR-29a*-deficient HSC function in vivo, we competitively transplanted 5 million total bone marrow cells from WT littermates, *miR-29a*$^{+/-}$, *Dnmt3a*$^{+/-}$, and *miR-29a*$^{+/-}$;*Dnmt3a*$^{+/-}$ mice into lethally irradiated congenic recipients each along with 5 million competitors. Peripheral blood donor cell chimerism was significantly higher in mice transplanted with compound *miR-29a*$^{+/-}$;*Dnmt3a*$^{+/-}$ cells compared with those transplanted with *miR-29a*$^{+/-}$ cells 6 months following transplant (Figure 7E). Mice transplanted with *miR-29a* Het cells exhibited significantly lower donor chimerism among immature hematopoietic cells, but *miR-29a*$^{+/-}$;*Dnmt3a*$^{+/-}$ HSCs demonstrated competitive long-term reconstitution capacity similar to that observed with WT donors when the recipient's BM and spleen were evaluated (Figure 7F; supplemental Figure 10C-D). We also evaluated HSC function from

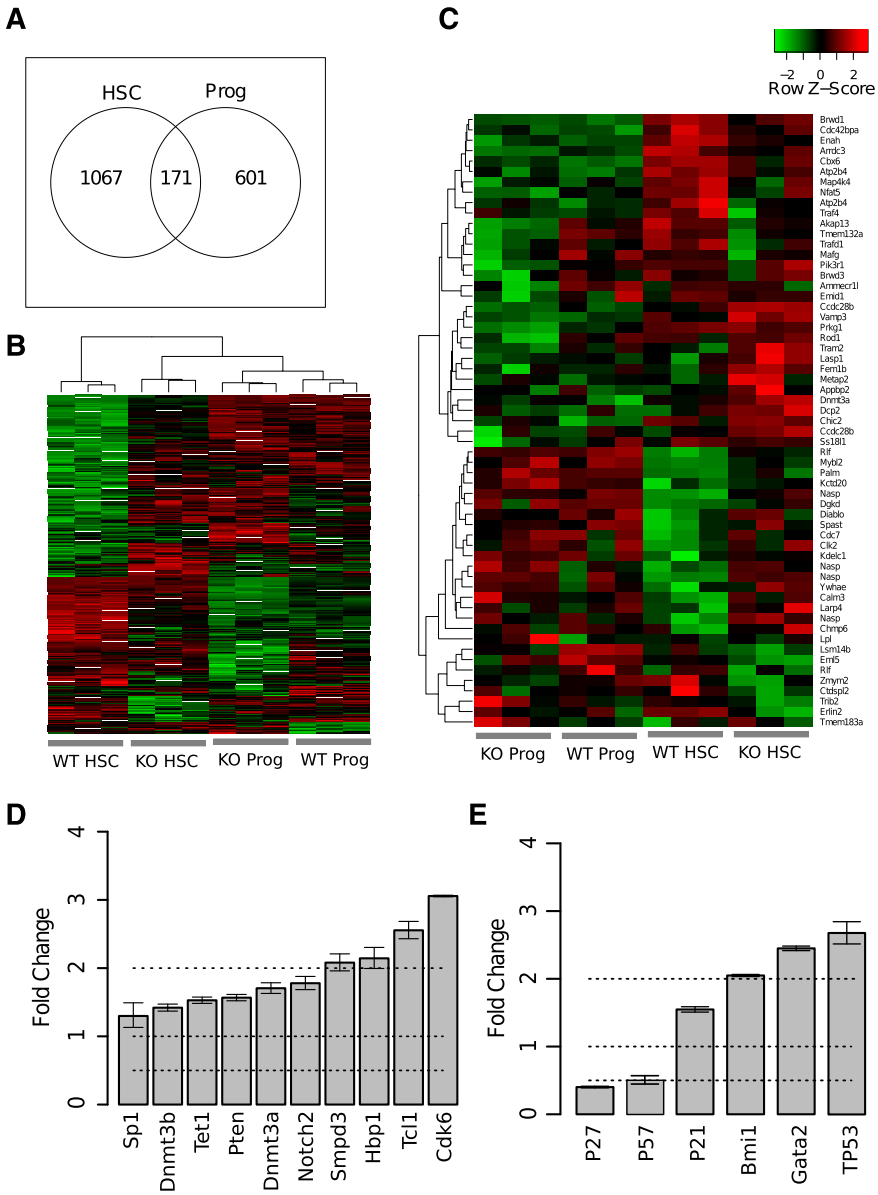

Figure 6. Gene expression profiling analysis reveals dysregulation of *miR-29a* targets in HSCs. (A) Numbers of differentially expressed genes in *miR-29a/b-1* KO or WT HSCs ($Lin^-c\text{-}Kit^+Sca\text{-}1^+CD34^-Slam^+$) or committed progenitor ($Lin^-c\text{-}Kit^+Sca\text{-}1^-$; Prog) cells. Genes were selected using a *P* value threshold of $<.05$ and a fold change >1.5. (B) Unsupervised clustering analysis of dysregulated genes identified in A. (C) Unsupervised clustering analysis of genes predicted to be targets of *miR-29a*. Predicted targets were identified using targetscan.org (Release 6.1). (D) Validation of changes in the expression levels of reported regulators of bone marrow HSPCs using c-Kit-enriched bone marrow cells from miR-*29a/b-1* WT or KO mice by qRT-PCR. Fold change was calculated by comparing KO with WT cells. The genes shown represent predicted *miR-29a* target genes with fold change >1 in *miR-29a/b-1* KOs compared with WT littermates. (E) Selected regulators of HSC function were chosen based on published genetic evidence. Fold change was relative to WT type. Error bars represent SEM from triplicate experiments.

mice expressing reduced levels of *miR-29a*, *Dnmt3a*, or both by competitive transplantation. Evaluation of the peripheral blood 16 weeks following transplant revealed that deletion of *Dnmt3a* increased the reconstitution capacity of *miR-29a*-null HSCs compared with WT HSCs, consistent with findings previously observed,[34] and this effect was slightly reduced in the setting of *miR-29a* heterozygosity. *Dnmt3a* heterozygosity resulted in a small, but statistically significant, increase in donor cell chimerism in the *miR-29a*-null background (supplemental Figure 11). Together, these data confirm that *Dnmt3a* is a critical downstream mediator of *miR-29a* function in HSCs.

Discussion

In this study, we assessed the role of *miR-29a* in hematopoiesis by evaluating mice lacking the *miR-29a/b-1* bicistron. Our studies reveal that *miR-29a* maintains HSC self-renewal and that *Dnmt3a* is a direct target of *miR-29a* that mediates the *miR-29a*-deficient hematopoietic phenotype. Thus, *miR-29a* is among a small but growing group of miRNAs shown to regulate HSC function. However, unlike other miRNA regulators of HSC function, which have been shown to regulate apoptosis or transforming growth factor-β signaling (*miR-125*),[12,14] PI3K/AKT/GSK3β signaling (*miR-126*),[15] or nuclear factor-κB signaling[16] (*miR-146*), *miR-29a* appears to largely mediate its effects on HSC function by regulating the epigenetic state of HSCs by directly inhibiting expression of the DNA methyltransferase enzyme, DNMT3a.

The *miR-29a/b-1*-null phenotype is specifically due to loss of *miR-29a* because only ectopic expression of *miR-29a*, but not *miR-29b,* restored both the in vitro colony forming and long-term reconstitution ability of *miR-29a/b*-1-deficient HSPCs. These findings are consistent with our previous studies in which we demonstrated that overexpression of *miR-29a*, but not *miR-29b*, is sufficient to induce myeloid leukemia by conferring self-renewal to committed myeloid progenitors.[9] Collectively, these findings provide further evidence that members of the same miRNA family may exert distinct biological functions. The biological basis of the unique effects of *miR-29a* and *miR-29b* in

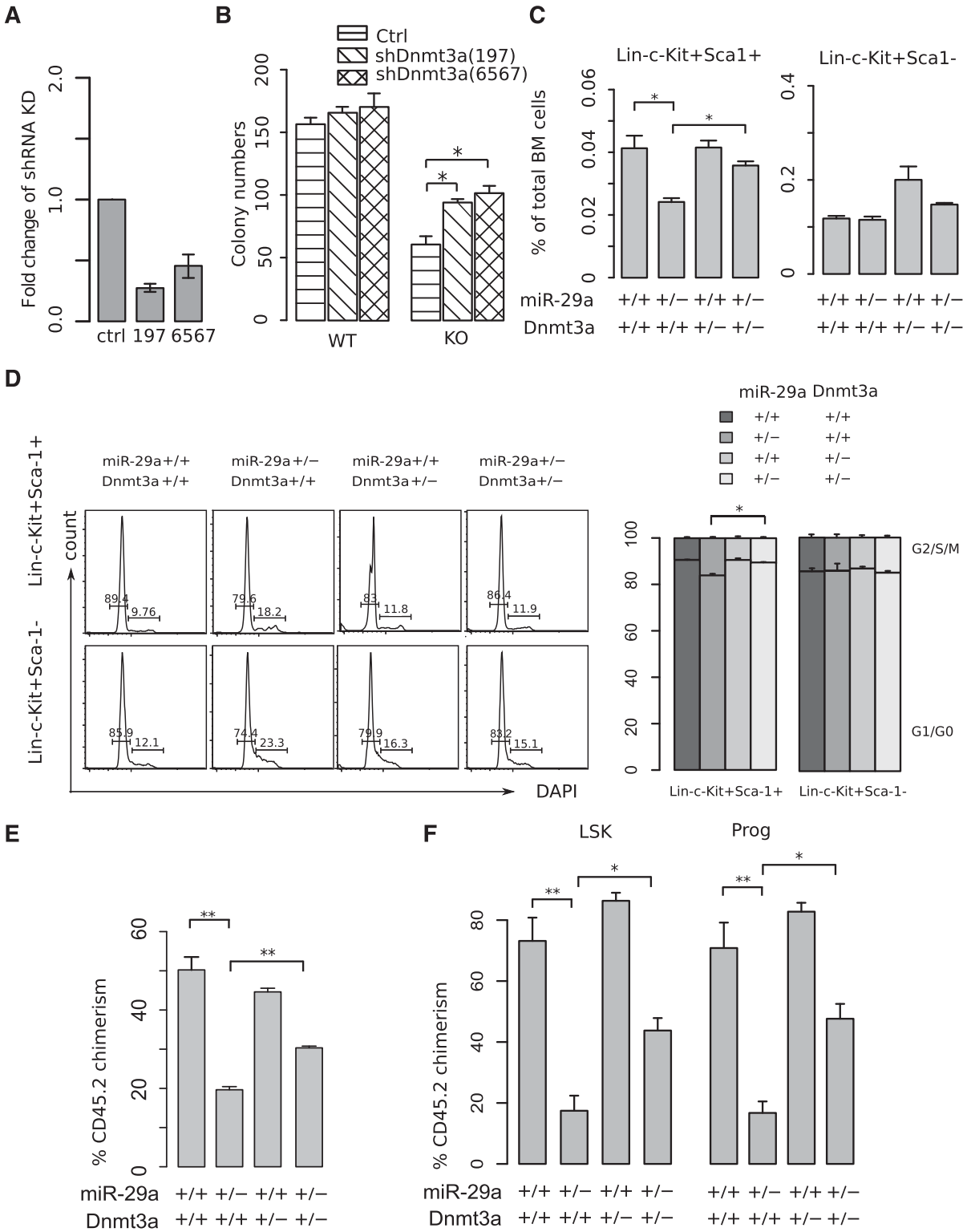

Figure 7. *Dnmt3a* dysregulation mediates *miR-29a/b-1*-deficient HSC defects. (A) shRNAs (197 and 6567) efficiently knock down *Dnmt3a* mRNA levels by qRT-PCR in NIH 3T3 cells. (B) Knock down of *Dnmt3a* partially restores *miR-29a/b-1*-null HSPC function as assessed by methylcellulose colony formation assay. Bone marrow cells were harvested from fluorouracil (5-FU) treated mice and infected with either empty GFP$^+$ retrovirus or shRNAs against *Dnmt3a*. GFP$^+$ cells were double-sorted after 2 rounds of infection and plated in 6-well plates with 200 cells per well, and colony numbers were counted at day 12. (C) The 3- to 4-week-old progeny from *miR-29a*$^{+/-}$ *Dnmt3a*$^{wt/fl}$[*Mx1*-Cre] mice were treated with poly I:C 3 times every other day and were analyzed 1 month following the last injection. LSK (left) cells were significantly increased in *miR-29a*$^{+/-}$*;Dnmt3*$^{+/-}$ mice, but committed progenitors were not affected (right). (D) Evaluation of cell cycle status of LSK cells in *miR-29a*$^{+/-}$, *Dnmt3a*$^{+/-}$, and *miR-29a*$^{+/-}$*;Dnmt3a*$^{+/-}$ mice using a DAPI DNA stain. Shown with representative flow cytometry analysis (left) and summarized results (right). (E) Five million total bone marrow cells from *miR-29a*$^{+/+}$*;Dnmt3a*$^{+/+}$, *miR-29a*$^{+/-}$*;Dnmt3a*$^{+/+}$, *miR-29a*$^{+/+}$*;Dnmt3a*$^{+/-}$, and *miR-29a*$^{+/-}$*;Dnmt3a*$^{+/-}$ mice were transplanted into lethally irradiated congenic recipients with 5 million competitor bone marrow cells. Peripheral blood donor chimerism was determined 4 months after transplant. (F) Bone marrow HSPCs were analyzed for donor cell chimerism. Error bars represent SEM from 5 to 12 mice for all experiments shown. Statistical significance was calculated using a Student *t* test: **P* < .05; ***P* < .01.

HSCs is not due to their tissue-specific expression, which has been shown for the thymus,[19] but likely due to a unique 3′ hexanucleotide nuclear localization sequence in *miR-29b*, which has been shown to enhance its turnover during nonmitotic portions of the cell cycle.[38]

Dnmt3a is a primary mediator of the *miR-29a*-deficient hematopoietic phenotype, as shRNA-mediated reduction of *Dnmt3a* activity in *miR-29a*$^{-/-}$ HSPCs partially restored their in vitro colony formation capacity. In addition, deleting one allele of *Dnmt3a* in *miR-29a/b-1*$^{+/-}$ mice restored HSC function, demonstrated by recovery of HSC frequencies and their long-term reconstitution capacity. These studies are consistent with prior studies demonstrating the essential role of DNA methyltransferases in maintaining HSC function; loss of *Dnmt3a* resulted in expanded HSC numbers, impaired HSC reconstitution activity, and defects in differentiation,[34,39] whereas ectopic *Dnmt3a* expression inhibited cell proliferation and survival.[34] Additional data supporting the physiologic relevance of *miR-29a* regulation of *Dnmt3a* come from gene expression studies, which show that both *miR-29a* and *Dnmt3a* are highly enriched in HSCs.[9,34] Thus, *Dnmt3a* is a physiologically relevant target of *miR-29a,* indicating that *miR-29a* regulates the balance between HSC self-renewal and differentiation/proliferation through a novel epigenetic mechanism. Nevertheless, the difference in HSC function between WT mice and *miR-29a*$^{+/-}$*; Dnmt3a*$^{+/-}$ mice suggests that other *miR-29a* targets likely contribute to the *miR-29a*-null phenotype. Possible candidates contributing to this phenotype include predicted direct *miR-29a* target genes revealed by the *miR-29a/b-1*$^{-/-}$ HSC transcriptome including *Cdk6*,[40] *Tcl1*,[41] and *Hbp1*.[9] Notably, *miR-29a*$^{-/-}$ HSCs exhibited dysregulated expression of numerous cell cycle regulators, reflecting their impaired ability to maintain quiescence, and it is likely that the observed increased cell cycling underlies, or at least reflects, the alteration in *miR-29a*$^{-/-}$ HSC function, as has been shown for other mouse mutants exhibiting increased HSC cycling and reduced function.[42]

We previously reported that *miR-29a* is expressed at high levels in human AML compared with purified HSPCs and that enforced expression of *miR-29a* in HSPCs is sufficient to drive a myeloproliferative phenotype and confer aberrant self-renewal to committed myeloid progenitors, supporting *miR-29a* as an important regulator of self-renewal.[9] However, it appears that *miR-29a's* function is cell context specific, as enforced expression of *miR-29a* or *miR-29b* suppresses AML cell proliferation and survival, providing a rationale for *miR-29b*-based therapies to treat AML patients.[32,43] Thus, because *miR-29a* is critical for maintaining HSC self-renewal and *miR-29* negatively influences AML cell growth, *miR-29* agonist therapies would be expected to provide simultaneous negative growth effects on AML cells while promoting HSC self-renewal. However, given the presence of preleukemic HSCs in AML patients,[44-46] whether or not such therapies may promote the growth or transformation of such preleukemic HSCs is of potential concern. Thus, examining the effect of *miR-29*-based therapies on preleukemic HSCs will be an important area of investigation in the future.

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Authorship

Contribution: W.H. designed and performed research, analyzed data, and wrote the manuscript; J.D., S.M., B.d.S., and A.L. contributed the mouse model, designed the research, and wrote the manuscript; L.C. contributed the Dnmt3a knockdown constructs; D.C. and C.E.M. helped generate and analyze DNA methylation data; S.S.C. designed the research and wrote the manuscript; and C.Y.P. designed research, contributed reagents, analyzed data, and wrote the manuscript.

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